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Antioxidants and redox signaling forum review**NADPH oxidases as regulators of tumor angiogenesis: current and emerging concepts**

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ABSTRACT

Significance: Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and peroxynitrite are generated ubiquitously by all mammalian cells and have been understood for many decades to inflict cell damage and to cause cancer by oxidation and nitration of macromolecules including DNA, RNA, proteins and lipids. **Recent advances:** A current concept suggests that ROS can also promote cell signaling pathways triggered by growth factors and transcription factors that ultimately regulate cell proliferation, differentiation and apoptosis, all of which are important hallmarks of tumor cell proliferation and angiogenesis. Moreover, an emerging concept indicates that ROS regulate the functions of immune cells that infiltrate the tumor environment and stimulate angiogenesis, such as macrophages and specific regulatory T cells. **Critical Issues:** In this review, we highlight that the NADPH oxidase family of ROS-generating enzymes are *the* key sources of ROS and thus play an important role in redox signaling within tumor, endothelial and immune cells to thereby promote tumor angiogenesis. **Future Directions:** Knowledge of these intricate ROS signaling pathways and identification of the culprit NADPH oxidases is likely to reveal novel therapeutic opportunities to prevent angiogenesis that occurs during cancer and which is responsible for the revascularization following current anti-angiogenic treatment.

INTRODUCTION: THE NEED TO UNDERSTAND NADPH OXIDASE-DEPENDENT SIGNALING IN CANCER

A substantial body of evidence indicates that one of the most important hallmarks of cancer is the chronic induction of angiogenesis - the process of new blood vessel formation (78, 79). This process allows solid tumors to grow beyond a few millimetres in diameter and then to metastasize and spread to distant sites. Therefore, there has been a keen interest in developing drugs to target angiogenesis for cancer therapy. The knowledge that pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF) are released from tumors and activate endothelial progenitors and vascular endothelial cells to proliferate and form neovasculature has given rise to the current inhibitors of angiogenesis (VEGF receptor blockers). Despite displaying some efficacy against cancers in both animal models (1) and in humans (54), the benefits of these compounds are short-lived with revascularization occurring, and initially responsive tumors progressing (18). Thus, there is a need for new drugs with sustained anti-angiogenic properties.

Two new paradigms in cancer research have emerged with tremendous potential to give rise to novel therapeutic strategies. The *first* proposes a novel role for reactive oxygen species (ROS) as a stimulus for tumor angiogenesis. The *second* suggests that angiogenesis and the revascularization following conventional anti-angiogenic drug therapy, is caused by *chronic inflammation* (18, 33, 127). ROS such as superoxide, hydrogen peroxide (H_2O_2), peroxynitrite ($OONO^-$) and hydroxyl radical (OH^\bullet) have been traditionally implicated in cancer as they can induce DNA damage and mutations, RNA alkylation, lipid peroxidation and protein damage and dysregulation (85). However, in addition to these direct cytotoxic and mutagenic effects, more recent evidence indicates that ROS are important regulators of signal-transduction pathways that promote angiogenesis and tumorigenesis. In this review, we will highlight the NADPH oxidase family of ROS-generating enzymes as key sources of ROS that

influence many facets of cell signaling during cell proliferation and apoptosis. Finally, we will draw attention to the novel concept that NADPH oxidase-derived ROS are crucial regulators of tumor anti-immunity *via* effects on specialized subsets of immune cells such as macrophages and T lymphocytes and that they could represent a novel molecular link between chronic inflammation and angiogenesis during cancer.

GENERAL FEATURES OF ROS

For many years ROS have been considered as by-products of aerobic respiration and mediators of a number of critical cellular functions such as the killing of pathogens. However, more recently ROS have been acknowledged as important signaling intermediates in processes such as cell proliferation, migration, differentiation and apoptosis. ROS are highly reactive oxygen-containing molecules occurring in both radical and non-radical forms. Superoxide, often considered the parent species of the ROS family of molecules, is produced during oxygen metabolism as a result of the one-electron reduction of molecular oxygen (Figure 1). The high reactivity of superoxide is due to the instability of an unpaired electron in its valence shell. Superoxide has been implicated in numerous pathological processes including cancer, cardiovascular disease (e.g. atherosclerosis and stroke) and acute and chronic diseases caused by microbial infections. Superoxide can directly damage DNA through oxidation (95), directly inactivate cellular antioxidants such as catalase and glutathione peroxidase (157), and induce cellular accumulation of sorbitol as well as activate pro-inflammatory NF- κ B (143). However, superoxide gives rise to additional ROS that possess different redox chemistries, and thus different physiological and pathophysiological effects. For example, superoxide is rapidly reduced, both spontaneously (59) and enzymatically (135) to give rise to H₂O₂ (Figure 1). Unlike superoxide, H₂O₂ has no net charge so, apart from being an efficient oxidant it is more lipid-soluble, with the potential to

diffuse through organelles and cellular membranes to sites distant from its production (144). Hydrogen peroxide modifies (1) cellular proteins *via* oxidation of sulphur-containing amino acids, cysteine and methionine (138, 156), (2) lipids by peroxidation (94) and (3) genetic material (i.e. RNA and DNA) (82). These oxidative processes have been implicated in many diseases in addition to cancer including Alzheimer's (137) and Parkinson's disease (122). However, perhaps the major detrimental properties of H_2O_2 , like superoxide, are in its ability to give rise to more reactive molecules. For instance, H_2O_2 can be reduced by transition metals, namely ferrous iron, with or without superoxide to form the highly reactive hydroxyl radical (OH^\bullet ; Figure 1) (100). The pathological formation of hydroxyl from H_2O_2 is a very important biological reaction. The hydroxyl radical is highly reactive and will indiscriminately oxidize the nucleotides of cellular DNA to cause breaks and lesions (for review see (25, 173)) which are processes linked to carcinogenesis (126). The oxidation of lipids by hydroxyl radical may influence many physiological processes and contribute to cellular dysfunction during cardiovascular disease (175).

Arguably one of the most important redox reactions occurring in biology is that between superoxide and the nitric oxide (NO) radical. With a reaction rate of $\sim 10^{10} \text{ MS}^{-1}$ it is one of the fastest reactions in biology giving rise to peroxynitrite ($ONOO^-$; Figure 1) (84). Peroxynitrite is an oxidising and nitrating agent that has been implicated in cancer (58) and other acute (99) and chronic (184) diseases. Through oxidation of cysteine and transition metal-containing proteins and nitration of tyrosine, peroxynitrite can influence the physiological properties of many proteins including haemoglobin, myoglobin, cytochrome c, inducible nitric oxide synthase (iNOS), endothelial NOS (eNOS), mitochondrial aconitase, phosphogluconate dehydratase, alcohol dehydrogenase, complexes I, II, III and IV of the electron transport chain and elicit changes in protein structure through thiol oxidation (147). In the artery wall, this reaction between superoxide and NO holds major biological

significance. First identified in 1987 (148), NO is regarded as one of the most important vascular signaling molecules owing predominantly but not exclusively to its properties as a vasorelaxant, anti-inflammatory and anti-thrombotic agent. The reaction of superoxide with vascular NO leads to endothelial dysfunction and vascular inflammation giving rise to hypertension (74) and atherosclerosis (197).

SOURCES OF ROS: NADPH OXIDASE FAMILY OF ENZYMES

Several enzymes produce superoxide and other ROS, including the mitochondrial electron transport chain, NOS, cytochrome P₄₅₀ reductase and xanthine oxidase. However, for all of these systems, superoxide production occurs as a by-product of another reaction that represents the main catalytic function of the enzyme/system or from a dysfunctional variant of the enzyme. In contrast, NADPH oxidases are the only enzymes whose primary function is to generate superoxide/ROS. NADPH oxidases are multi-subunit enzyme complexes highly conserved across a vast array of organisms (106) with significant homology among higher order mammals (29). The family is comprised of 7 oxidases, named Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2 (50, 166).

General features of Nox subunits

The Nox-containing NADPH oxidases (Nox1-5) are highly proficient ROS-generating enzymes with a wide distribution among human tissues (103). The catalytic Nox protein contains six hydrophobic transmembrane domains and a long intracellular C-terminus tail. The transmembrane domains contain four conserved histidine residues, which bind heme groups believed to be essential for electron transfer across the plasma membrane (20). The electrons utilized by the oxidase are donated from the oxidation of NADPH or NADH. The cytosolic C-terminus contains putative NADPH binding sites which transfers electrons from

NADPH to a flavin adenine dinucleotide (FAD) bound at the N-terminus end of the cytosolic tail (77, 116). The electrons are then transferred through the heme groups to reduce molecular oxygen, forming superoxide or H₂O₂, depending on the Nox isoform.

In addition to the catalytic Nox protein, Nox1-4 require a number of other regulatory proteins which are important for enzyme localization, stability and activation, including p22phox, p47phox, p67phox, p40phox and Rac (145) (Figure 2). Nox5 does not require interaction with other regulatory proteins but is regulated by calcium. Although the Duox proteins have been shown to interact with p22phox, the activity of the enzymes are not influenced by this interaction and it remains unclear whether additional proteins are required for activation (194).

In the following sections we provide a brief review of some key structural features of each of the Nox oxidases and their respective regulatory subunits (Figure 2). For a more comprehensive description we refer you to the following recent reviews (115, 165).

NADPH oxidase 2 (Nox2 oxidase)

The Nox2 subunit is bound within cellular membranes including plasma and endoplasmic reticulum to a second protein, p22phox, in a heterodimer named cytochrome b-558 (149). The stable expression of a mature Nox2 and the activity of the Nox2 oxidase is dependent on p22phox co-expression (93, 149) with Nox2 monomer rapidly targeted for degradation by cytosolic proteasomes in p22phox deficiency (42). In addition to cytochrome b-558 stabilisation, the C-terminus of p22phox contains a region of high proline content that interacts with a Src homology 3 (SH3) domain of the organizer protein p47phox; a process which is essential for oxidase activation (114). p47phox is a cytosolic protein which translocates upon stimulation to the membrane bound cytochrome b-558 heterodimer. The p47phox protein has two regions with significant SH3 homology, one of which is bound to an

upstream proline-rich region of the protein under basal conditions. In response to phosphorylation of a number of serine residues on p47phox, the SH3 domains become exposed allowing interaction of the first SH3 domain with the p22phox proline-rich region (56). Additionally p47phox can interact with at least two other binding sites on the Nox2 protein (44). p47phox is essential for the recruitment of another cytosolic protein p67phox to the cytochrome b-558 complex (81). p47phox interacts with p67phox *via* the second SH3 domain (40). Upon translocation to the membrane, p67phox directly interacts with Nox2 *via* an activation domain (76) and binds a small GTPase, Rac (47). Finally, p40phox interacts with the Nox2 complex *via* binding domains on p47phox and p67phox, and although it has been shown to promote Nox2 oxidase activity it is not considered an essential regulator (4, 60). Nox2 oxidases are ubiquitously expressed in mammalian cells. They are highly expressed in inflammatory cells such as macrophages, and are readily detected in endothelial cells.

Nox1 oxidase

Almost 25 years after the discovery of NADPH oxidases in phagocytic cells, the first homologue of the gp91phox subunit of the prototypical phagocytic oxidase was identified and termed Mox1 (for mitogen oxidase 1) (178). Later renamed Nox1, this protein shares 56% homology with Nox2 and is similar in many respects, structurally to Nox2. The structural similarities between Nox1 and Nox2 include the C-terminal cytoplasmic tail containing the NADPH binding sites, FAD moieties and 2 heme regions as well as 6 putative transmembrane spanning regions. Like Nox2, Nox1 is complexed and stabilised with p22phox at the protein level (7, 93) and is tightly and efficiently regulated by several modulatory protein subunits. Indeed, Nox1–p22phox heterodimers are relatively inactive but produce superoxide in the presence of NoxO1 (Nox organiser 1) and NoxA1 (Nox activator 1), which are homologous to p47phox and p67phox, respectively (6, 12, 67). It is noteworthy that both p47phox and

p67phox are capable of stimulating superoxide production in Nox1-expressing systems and but not as efficiently as NoxO1 and NoxA1 (6, 67). Nox1 is expressed in colon and airway epithelium, stomach uterus, as well as vascular smooth muscle cells (12, 29, 178).

Nox3 oxidase

Nox3 was first described about 10 years ago based on its sequence similarity to other Nox isoforms (96). Its overall structure is similar to that of Nox1 and Nox2, in terms of transmembrane domains, the length of the extracellular loops, NADPH- and FAD-binding sites, and the localization of the heme-coordinating histidines (29, 96). Nox3 is also dependent on p22phox for its stabilization, plasma membrane localization and activation (185). Activation of Nox3 occurs in the presence of NoxO1 and NoxA1, although some evidence is contradictory. In heterologous expression studies, p47phox and p67phox are capable of activating Nox3 (13, 185, 186); however, the physiological relevance of this is unknown. The dependence of Nox3 on Rac is also still a matter of debate. Overall the Nox3 oxidase is constitutively active and its tissue distribution, as determined by real time PCR and in situ hybridization, demonstrates a very high expression in the inner ear, including the cochlear and vestibular sensory epithelia and the spiral ganglion (13). Low levels of Nox3 can also be detected in other tissues, including fetal spleen (96), fetal kidney (13, 29) skull bone, and brain (13).

Nox4 oxidase

First identified in kidney and initially termed 'Renox' (66, 169), Nox4 shares 39% homology with Nox2 and possesses the same conduit of electron transporting moieties as Nox1 and Nox2, including the NADPH binding site, FAD and heme groups. Like Nox1 and Nox2, the prototypical, full size Nox4 forms a heterodimer with p22phox, which is necessary for its

activity and stability (7, 131). However, unlike Nox1 and Nox2, the PRR region of p22phox is not necessary for Nox4 oxidase activity (93). Thus, the additional molecular interactions that p22phox makes with organiser proteins like p47phox and NoxO1 are not crucial for Nox4 oxidase-dependent ROS production (93). As a consequence, Nox4 may serve as an important constitutively active ROS-generating system, whose overall ROS output is governed by its expression level (200), post-translational modification and via its interactions with the polymerase δ -interacting protein 2 (POLDIP2) (123). Contrary to Nox1, Nox2, Nox3 and Nox5, Nox4 activity results in the release of H_2O_2 rather than superoxide (181). This is most probably owing to a delayed dissociation of superoxide from the catalytic subunit due to hindrance by the third extracytoplasmic loop, allowing time for a second molecule of superoxide to be formed and to be consequently dismutated before being released from the enzyme (181). Nox4 is expressed in kidney cortex in high amounts as well as endothelial cells, smooth muscle cells, heart, pancreas and osteoclasts but not in phagocytic cells (for review see (50, 165).

Nox5 oxidase

Nox5 contains a unique, cytosolic N-terminal Ca^{2+} binding domain with calmodulin-like EF-hand motifs that render this oxidase highly sensitive to Ca^{2+} (14) and to phosphorylation that increases Ca^{2+} sensitivity (88). However, it also possesses the same redox centres and NADPH binding sites as Nox1 to Nox4. Nox5 displays only ~22–27% homology to Nox1 through to Nox4 (14), but unlike those Nox proteins, it does not require p22phox for activation (93) nor any of the other known regulatory subunits. Interestingly, there are five splice variants identified to date, named Nox5 α , Nox5 β , Nox5 γ , Nox5 δ and Nox5s, that differ in the sequence of their Ca^{2+} binding regions as well as their tissue distribution (16). Nox5s

lacks the Ca^{2+} binding regions and is constitutively active (16). Nox5 is expressed in human lymphoid tissues, testis and spleen (14) and in endothelial cells (16).

Dual oxidase (Duox)

Duox1 and 2 are *dual property* enzymes, which comprise a catalytic protein containing an N-terminus peroxidase domain and a C-terminus NADPH oxidase region. They are largely localized to the thyroid gland and lung epithelial cells of adults where they are thought to be involved in hormone biosynthesis and host defence through the production of H_2O_2 by the NADPH oxidase domain (39, 51, 68). The role of the peroxidase region of the Duox is not well understood but has been shown to promote tyrosine-dependent cross linking of structural proteins in *C. elegans* (49).

CELLULAR LOCALIZATION AND FUNCTIONS OF NADPH OXIDASES IN THE TUMOR ENVIRONMENT

ROS-generating cells relevant to tumor development and progression, but not strictly limited to angiogenesis, include: *tumor cells*, *endothelial cells* (of both blood and lymphatic vessels) and immune cells such as macrophages and T lymphocytes that infiltrate the tumor environment. In the following section we provide a summary of the current state of knowledge of Nox oxidase expression and activity in tumor and endothelial cells, and the cell signaling mechanisms they regulate to influence proliferation, apoptosis and angiogenesis during cancer (Figure 3). In the last section on “Emerging concepts” we will put forward novel ideas on Nox-dependent regulation of lymphangiogenesis, and of immune cell function, particularly of macrophages and immunoregulatory T lymphocytes.

Tumor cells: NADPH oxidase-derived ROS regulate autocrine and paracrine effects

Nox1, Nox2 and Nox4 are known to be expressed in multiple tumor cell types. For example, Nox1 is highly expressed in human colon cancer cells (110) and prostate cancers (119). Nox4 is expressed in human gliomas (170), melanomas (23), pancreatic adenocarcinomas (192), renal cell carcinomas (130) and ovarian cancer cells (202). Nox2 is expressed in myeloid leukemia cells and prostate cancer cells (118). Tumor cells produce substantial amounts of ROS (11, 75, 178, 179) via NADPH oxidases that possess autocrine effects, such as promoting their own proliferation, most likely through regulation of proliferative signaling kinases such as extracellular-signal-regulated kinases (ERK)-1/2 and cell survival factors like Akt. Also, Nox1-dependent ROS production critically mediates the effects of the oncogene Ras, in particular in the Ras dependent upregulation of VEGF that occurs via the Raf-MEK-ERK pathway (98). Moreover, Nox1-dependent superoxide production occurring in tumor cells may activate Src and thereby stimulate downstream phosphatidylinositol 3-kinases (PI3K) and ERK-dependent survival pathways in response to angiopoietin-like-protein 4 (ANGPTL4) that confers resistance to anoikis (212).

Although ROS generated by tumor cells are likely to act in an autocrine fashion to influence cell proliferation/survival and other cellular processes, ROS have also been shown to regulate the release and actions of tumor-derived growth factors that induce endothelial cell proliferation leading to angiogenesis. For instance, ROS production within tumor cells dramatically promotes release of paracrine growth factors such as VEGF, which in turn stimulate proliferation, migration and tube formation in nearby endothelial cells. Indeed, ROS were found to increase VEGF expression in tumor cells and to also promote the expression of its receptor, VEGF receptor-1 (VEGFR-1) (8). In PC-3 prostate cancer cells, NADPH oxidase-derived ROS stimulates hypoxia inducible factor (HIF)-1 α , which in turn promotes the release of VEGF-A and subsequent angiogenesis. Thus, given that tumor-derived ROS are

an important trigger of angiogenesis, identifying the source of ROS in tumors under these circumstances may reveal novel targets to suppress ROS production for anti-angiogenesis therapy. In addition, NADPH oxidase expressed within endothelial cells is crucial for the ability of growth factors such as VEGF released by tumors to stimulate endothelial cell proliferation and consequently angiogenesis.

Endothelial cells: NADPH oxidases in cell proliferation and apoptosis

It has been known for some time now that ROS, including H₂O₂ and superoxide, can function as signaling molecules in vascular endothelial cells (189). Endothelial ROS are produced upon stimulation by growth factors such as VEGF, transforming growth factor- β (TGF- β), fibroblast growth factors and angiopoietin-1, cytokines (i.e. tumor necrosis factor- α ; TNF- α), fluid shear stress, ischemia–reperfusion, and leukocyte adhesion (83, 108, 132, 205). In endothelial cells, the Nox catalytic subunits Nox1, Nox2, Nox4, Nox5, and regulatory subunits Rac1, p47phox, p67phox and p22phox are expressed at the mRNA and protein level to varying degrees and with species variability. Ago *et al.*, (2004) and Pendyala *et al* (2009) showed that the mRNA expression level of Nox4 was markedly higher than Nox1, Nox2 and Nox5 in human pulmonary artery endothelial cells, human lung microvascular endothelial cells and cultured human umbilical vein endothelial cells (5, 24, 150). In human endothelial cells, Nox5 is highly expressed and appears to represent a quantitatively relevant source of ROS in these cells. However, Nox5 is not expressed in rodent tissues.

Evidence supporting a role for ROS produced by NADPH oxidase in the proliferation of endothelial cells came from studies that showed expression of p22phox, which is essential for the activities of Nox1, Nox2 and Nox4 was substantially higher in proliferating endothelial cells than in quiescent cells (15). Furthermore, transfection of cells with antisense for p22phox dramatically reduced both ROS production and cell proliferation (15). Evidence

for Nox isoform-specific effects on endothelial cell proliferation has come predominantly from siRNA experiments. Knockdown of Nox4 with siRNA attenuated hyperoxia-induced cell migration and capillary tube formation, suggesting that ROS generated by this NADPH oxidase isoform regulates endothelial cell motility and their migration (150). Furthermore, overexpression of Nox4 in endothelial cells increases ROS production and stimulates cell proliferation, whereas siRNA silencing of Nox4 has the opposite effect (38, 151, 152, 164) .

Nox2 is a critical source of ROS in endothelial cells and an important regulator of their function (28, 73). In humans, the genetic deficiency of Nox2 (i.e. in chronic granulomatous disease (CGD) patients) is associated with enhanced endothelium-dependent flow-mediated vasorelaxation and decreased markers of vascular aging and oxidative stress by limiting nitric oxide availability (193). Using isolated vessels from wild-type and Nox2-deficient mice, it has been demonstrated that superoxide formed by endothelial Nox2-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension by scavenging endothelium-derived NO (91). Moreover, endothelial Nox2 underpins vascular oxidative stress and contributes to early atherosclerotic plaque formation in the hypercholesterolemic mouse model of atherosclerosis (90). Thus, Nox2 has a dual function in the vasculature where it is involved in normal physiological signal transduction, but also in generation of pathological levels of ROS, which contribute to oxidative stress in various cardiovascular diseases (50, 166). Nox2 is also involved in endothelial cell proliferation, as knockdown of Nox2 with siRNA affects endothelial cell morphology and is necessary for cell survival (86, 151). Nox2 also tethers with actin cytoskeleton via IQGAP1 at the leading edge of endothelial cells to direct ROS production and their migration (86). Rac1, which regulates Nox2 activity (but not Nox4 activity), is localized at the leading edge in wound-induced migrating cells (104) and endogenous H₂O₂ accumulates at the membrane ruffles in actively migrating endothelial cells (86). Overexpression of the active (GTP-bound) form of Rac1

induces loss of cell–cell adhesion (191) and cytoskeletal reorganization in primary human endothelial cells (139) through increased H₂O₂ production, which are required for endothelial cell migration. In addition to Rac1, during directed cell migration, p47phox translocates from the perinucleus to the membrane and to the leading edge or focal complexes through binding in endothelial cells (201).

Nox1 also plays an important role in endothelial cell proliferation, sprouting and migration (64), whereas in sinusoidal endothelial cells, Nox1 regulates apoptosis (97). Nox5 variants were found to be actively involved in the generation of ROS, proliferation and formation of capillary-like structures in human microvascular endothelial cells (16), however, much less is known about the role of Nox5 in general, largely due to the fact that Nox5 is not found in rodents (92).

CELL SIGNALING MECHANISMS AND PATHWAYS INFLUENCED BY NADPH OXIDASE-DERIVED ROS

Proliferative kinases

Under physiological conditions, Nox2 and Nox4 are important intermediates in signal transduction pathways regulating the activities of a large panel of growth factors, cytokines and hormones. As both Nox2 and Nox4 regulate endothelial cell proliferation, it is the specific localisation of these enzymes that is likely to be critical for their respective roles in activation of cellular signaling pathways. The association of Nox2 and its regulatory subunits p47phox, p67phox and p22phox to the cytoskeleton can affect the re-arrangement of the endothelial cytoskeleton during cell proliferation and migration (3, 86). In contrast to Nox2, localisation of Nox4 to the peri-nuclear and endoplasmic reticulum membranes (5, 24, 71) could conceivably result in activation of kinases such as mitogen-activated protein kinase (MAPK) and Src to promote cell proliferation.

MAPK families play an important role in complex cellular functions like proliferation, differentiation, development, transformation and apoptosis. At least three MAPK families have been characterized: ERK, Jun kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK (reviewed in (211)). ERK1 and ERK2 regulate endothelial cell proliferation and migration during mouse embryonic angiogenesis (174). Furthermore, MAPK regulates many aspects of adult endothelial cell activation including survival (62, 206), proliferation and migration (31). Buul *et al* (2005) found that on endothelial monolayers, Rac mediates ROS production *via* Nox2 and thus leads to the activation of p38 MAP kinase and loss of monolayer integrity through the inactivation of VE-cadherin-mediated cell–cell adhesion (24). Similarly, this pathway is also activated following leukocyte adhesion, and it is important for efficient leukocyte transendothelial migration. Conversely, in isolated or freshly-seeded endothelial cells, ROS are required for spreading and formation of cell–cell contact (24). This latter finding may be partially explained by inefficient inactivation of Rho, for which ROS are also required (142). Peshavariya *et al* (2009) showed that Nox2 oxidase-dependent superoxide production is anti-apoptotic by regulating the morphology of the cytoskeleton in an Akt-dependent manner (151). Basal Nox4 oxidase-dependent ROS alters the activity of MAPK (24, 71, 89, 136, 152) and Nox4 overexpression and formation of an active complex with p22phox enhances superoxide formation and phosphorylation of p38 MAPK(71) in endothelial cells. Furthermore, ROS, most likely H₂O₂, are generated by Nox4 *via* the activation of ERK1/2 and Akt (151). In addition, activation of p38 MAPK has been also implicated in induction of cell apoptosis via Nox4 activity (188).

The tyrosine kinase Src plays an important role in hyperoxia-induced tyrosine phosphorylation of p47phox and NADPH oxidase activation in human pulmonary artery endothelial cells (30). The ERK and p38 MAPK pathways also contribute to NADPH oxidase activation in these cells (71, 89). ROS are important for VEGF-induced Src activation and

Akt, contributing to the stimulation of angiogenesis in endothelial cells (120, 190). It is therefore clear that ROS, derived from the activities of Nox2 and Nox4, play important roles in the activation of a number of endothelial cell signaling pathways, and as such are likely to be major contributors to tumor angiogenesis.

Angiogenic growth factors and transcription factors

ROS are important players in cancer biology (121). In tumor endothelial cells, Nox enzymes contribute to angiogenesis through ROS-dependent mechanisms as described above, and thereby promote tumor growth *via* activation of signaling pathways. A number of transcription factors and genes involved in tumor angiogenesis are regulated by ROS, including VEGF-A, HIF-1, p53, and matrix metalloproteinases (MMPs).

VEGF: The mitogenic and chemotactic effects of VEGF-A in endothelial cells are mediated mainly through VEGFR-2, which is activated through autophosphorylation of tyrosine residues in the cytoplasmic kinase domain (172). This event is followed by activation of downstream signaling pathways such as MAPK, Akt and eNOS, which are essential for endothelial cell migration and proliferation (107). VEGF-A stimulation increases ROS production via activation of Rac1-dependent NADPH oxidase in endothelial cells (190, 204, 213). Zhuang *et al* (2010) found that overexpression of Nox4 significantly enhances VEGF-A-induced ROS generation and cell adhesion molecules in endothelial cells (213).

HIF-1: High expression levels of the transcription factor HIF-1 are observed in many human cancers (63, 134, 203). In addition to modulating factors involved in angiogenesis, HIF-1 activates the transcription of many genes involved in other aspects of tumor growth including cancer cell survival and invasion (37, 63, 134), and its levels correlate with tumorigenesis

(134). In response to tumor hypoxia, HIF-1 up-regulates a number key angiogenesis-related genes including VEGF-A and erythropoietin (117, 195). The Rac1/Nox/ROS pathway plays an important role for up-regulation of HIF-1 and VEGF-A expression in response to hypoxia (61). These reports suggest that ROS play an important role in upregulation of HIF-1 protein expression, which in turn contributes to upregulated VEGF-A expression and subsequent tumor angiogenesis.

p53: p53 is an important intracellular mediator of the stress response, including that initiated by ROS, and is now also recognized as a modifier of the angiogenic response. Salmeen *et al* (2010) found that ROS derived from Nox2 (but not Nox4) are functionally involved in the regulation of the cell cycle inhibitors p21^{cip1} and p53 and participate in endothelial cell cycle regulation and apoptosis (160). p53 interacts with the HIF system and Nox, but also has direct effects on angiogenesis regulators by interfering with translation mechanisms of angiogenesis factors and mediators such as VEGF-A (210) .

MMPs: MMPs are another family of enzymes involved in the progression of tumor-induced angiogenesis whose activities are regulated by ROS (146). MMPs regulate endothelial cell ingression into the tumor tissue by degrading extracellular matrix and freeing the way for migrating endothelial cells (69). ROS can activate MMP-10 via VEGF-A (80). Furthermore, VEGF-A and NO stimulate MMP-1 and 2 expression in endothelial cells (69). A recent study showed that the pro-inflammatory cytokine TNF- α , stimulates H₂O₂ production that promotes p38 MAP kinase and MMP-9 activation as well as sprouting angiogenesis in endothelial cells most likely *via* activation of Nox2 (154).

STUDIES IN TRANSGENIC AND KNOCKOUT MICE SHOW PROMISE BUT *IN VIVO* EVIDENCE IS STILL LACKING

Our review of the literature above is in line with the notion that ROS derived from NADPH oxidase play an important role in VEGF-A signaling linked to angiogenesis in endothelial cells. NADPH oxidase appears to be localized within discrete subcellular compartments, thereby activating specific redox signaling pathways. These events could be regulated by VEGF, HIF-1, p53 or MMPs, which contribute to temporal and spatial organization of ROS-dependent VEGF-A signaling linked to angiogenesis in endothelial cells. However, despite considerable amount of *in vitro* evidence that Nox oxidases expressed in endothelial cells are likely to influence tumor angiogenesis, close inspection of the literature reveals that there is still very little evidence to support this *in vivo* using tumor mouse models and key cell-specific Nox knockout mice. In fact, to the best of our knowledge the recent study by Garrido-Urbani *et al* (2011) using Nox1^{-/-} mice is the only study so far to have utilized any strain of Nox knockout mice in the study of tumor angiogenesis (64). Implantation of tumorigenic B16F0 melanoma cells subcutaneously in wild-type mice resulted in vascularised tumors after 10 days (64). In Nox1^{-/-} mice, the tumors that had developed were smaller and significantly less vascularised. This reduction in angiogenesis was associated with a reduction in expression of several genes, including VEGF, MMP-2 and MMP-9, and a reduction in NF-κB activity.

Although there is no *in vivo* evidence yet that Nox2 promotes tumor angiogenesis from the use of Nox2-deficient or -transgenic mice, there is certainly substantial evidence implicating Nox2 in ischemia reperfusion-dependent angiogenesis. Studies utilizing sponge implant assays demonstrate that VEGF-induced angiogenesis is significantly reduced in wild-type mice treated with the antioxidant N-acetylcysteine (26) and also in untreated Nox2^{-/-} mice, suggesting that ROS derived from Nox2 activity play an important role in angiogenesis

in vivo (190). Also, neovascularization was significantly impaired in Nox2^{-/-} mice as compared with wild-type mice in an ischemic hindlimb model of angiogenesis (183, 187). To date, only one study has reported a role of Nox4 in the endothelial response to *tumors in vivo* (19). Bhandarkar et al (19) used a model of human hemangioma to implicate a link between Nox4 and tumorigenic angiogenesis. The role of Nox4 has been established in alternative models of angiogenesis, such as that occurring following hypoxia. Hypoxia is associated with upregulation of Nox4 in the endothelium, and promotion of the angiogenic process including endothelial proliferation, migration, and tube formation (34). Importantly, endothelial-specific Nox4 overexpression *in vivo* led to enhanced angiogenesis in response to hypoxia. Clearly, there is a huge knowledge gap in our understanding of the regulation of tumor angiogenesis *in vivo* and future studies should utilize mice with cell-specific inhibition of Nox oxidases.

EMERGING CONCEPTS

NADPH oxidase and lymphangiogenesis?

Lymphangiogenesis is observed in many physiological and pathological settings, including embryonic development, tissue regeneration, wound healing and certain cancers. Similar to angiogenesis, we (208) and others (48, 72) have found that the VEGF family regulates lymphangiogenesis *via* VEGFR-2/VEGFR-3 homodimers or VEGFR-2-VEGFR-3 heterodimers. The presence of VEGFR-3 correlates with lymph node metastasis in many solid tumors, including prostate (207, 209), small (22) and non-small lung carcinoma, leukemia (45), skin (124), gastric (168) and breast carcinoma (198). As many of the VEGF receptor signaling pathways activated by VEGF-A in endothelial cells, including MAPK, Src and PI3K/Akt, are similarly activated in lymphatic endothelial cells (101, 125, 133), it is likely that ROS may play a role in the regulation of lymphatic vessels. Hypoxia was shown to increase the expression levels of transcripts encoding VEGF-C, VEGF-D, as well as their

receptor VEGFR-3 in endothelial cells (141). Furthermore, Schoppmann *et al* (2006) showed that HIF-1 correlates with VEGF-C expression and lymphangiogenesis in breast cancer (162). Tumors with high levels of HIF-1 α expression had a significantly increased amount of tumor-associated lymphangiogenesis. Indeed, HIF-1 α may be a key factor regulating tumor-induced lymphangiogenesis as inflammation and VEGF-C-expressing inflammatory cells have both been shown to play a crucial role in tumor-associated lymphangiogenesis (33, 161). Irigoyen *et al* (2007) performed genome-wide analysis of hypoxia-dependent regulation of gene expression in lymphatic endothelial cells and found that in a low oxygen environment Nox4 levels were increased (by 2-fold), along with other oxidative stress genes such as SOD2 (2.7-fold) and ARG2 (3-fold) (87). The study did not analyse or report expression of any other NADPH oxidase family members. We speculate that lymphatic endothelial cells are likely to be regulated via NADPH oxidases akin to the observations in blood vessel endothelial cells.

Are immune cell NADPH oxidases perpetrators of chronic inflammation-induced angiogenesis?

There is a growing appreciation that inflammation plays a central role in angiogenesis and in re-vascularization in many cancers (33, 127). As a consequence, Mantovani and colleagues (2010) have recently suggested that inflammation should be recognized as an important hallmark of cancer (128). Epidemiological data supports this hypothesis (for review see (128)) identifying an association between inflammation and cancer initiation. For example, in humans, persistent inflammation caused by microbial infections such as *Helicobacter pylori* are associated with gastric cancer, colonization with the bacterium *Haemophilus influenzae* may lead to increased lung cancer risk and, via unknown pathogens, prostatitis is associated with prostate cancer. Key features of cancer-related inflammation include the infiltration of inflammatory cells such as macrophages and T cells, particularly T regulatory

cells (33, 55, 127). Animal and clinical studies provide strong evidence that tumor-associated macrophages promote tumorigenesis. Meta-analyses demonstrate that in over 80% of such studies there is a correlation between macrophage density and poor patient prognosis (21). For example, there is a strong association between poor survival and increased macrophage density in breast, prostate, thyroid, lung and liver cancers (for review see (112, 113, 153)).

Macrophages are central to many immune responses and are immunoregulatory cells within the tumor. Macrophages are a dynamic and heterogeneous type of inflammatory cell, which show remarkable plasticity and exist in a number of phenotypically distinct states governed by stimuli within their immediate environment. Macrophages are now generally classified broadly into two categories, i.e. the '*classically activated*' M1 macrophages and the so-called '*alternatively activated*' M2 macrophages, based on their respective profiles of cytokine production, gene expression and cell surface markers (17, 129). The M1 macrophages secrete pro-inflammatory mediators, including interleukin (IL)-1 β , IL-18, TNF- α and IL-6, and express genes such as iNOS. Importantly, M1 macrophages are powerful generators of ROS such as superoxide, nitric oxide and peroxynitrite. The M2 macrophages in contrast, produce anti-inflammatory cytokines such as IL-10 and release angiogenic factors such as VEGF and TGF- β .

The initiation and progression of angiogenesis, and re-vascularization following VEGF inhibition can be the result of the infiltration/recruitment of macrophages to the tumor (32). The persistent recruitment of monocytes/macrophages in the tumor microenvironment activates them to release ROS and a wide variety of growth factors and cytokines including TGF- β and VEGF, all of which exacerbate the mutagenic environment, and in doing so stimulate angiogenesis (32). Thus, it is conceivable that angiogenesis and tumor progression could be promoted by ROS generated by macrophages (Figure 3). In actuality, NADPH oxidase was first identified in inflammatory cells, such as macrophages and neutrophils, as

the enzyme responsible for the respiratory burst essential for the microbicidal function of these cells (10, 159). The NADPH oxidase complex here consists solely of Nox2 and on a molar basis these cells generate by far the most superoxide of all mammalian cells. Thus, it would not be surprising if macrophage Nox2 oxidase plays a key role in tumor angiogenesis.

Does NADPH oxidase regulate immunosuppressor cells such as Tregs?

CD8⁺ and CD4⁺ T lymphocyte subsets that infiltrate the tumor are a crucial component of the adaptive immune system that mounts an anti-tumor response. They are present in high numbers in solid tumors and their presence correlates with a better prognosis in several types of cancers including ovarian, pancreatic, breast and prostate cancers. This is particularly the case for cytotoxic CD8⁺ T lymphocytes that are generally considered to be tumorlytic. The role of CD4⁺ T cells, however, is more controversial. In particular, an increased abundance of a subset of CD4⁺ T cells that co-express CD25 and the transcription factor FoxP3⁺, (otherwise known as regulatory T cells or Tregs) in the circulation and in the tumors of patients with ovarian cancer, gastrointestinal malignancies, breast cancer, pancreatic cancer and prostate cancer is predictive of a poor survival (36).

Tregs are immunosuppressor cells, which play an essential role in controlling immune responses to either self or non-self antigens. They are considered to be associated with poor prognosis because they suppress CD8⁺ and CD4⁺ effector T cell proliferation and function to thereby limit their anti-tumor effects. Despite the well-known immunosuppressive function of Tregs, the mechanisms by which they achieve this are likely to be numerous, and are still unclear. The best understood pathway involves the release of TGF- β and IL-10, both of which possess immunosuppressive effects. Given the inherent redox sensitivity of T cells, a recent study hypothesized that ROS may mediate the direct suppression of effector T cells by Tregs (52). Indeed, Tregs from p47phox^{-/-} mice have a reduced capacity to suppress effector T cells

compared to that of wild-type Tregs (52). Moreover, effector T cells from p47phox^{-/-} mice were significantly less sensitive than wild-type effector T cells to suppression by exogenous TGF- β (52). In addition, Nox2 oxidase may regulate the differentiation of Treg and effector T cells both *in vitro* and *in vivo* (111). Lee *et al* (2011) showed that Nox2 deficiency decreased the development of Tregs, but significantly promoted development of T effector cells such as Th1, Th2 and Th17 cells (111). Finally the activation of Tregs may be partially attributed to the actions of Nox2 expressed in macrophages (102). *In vitro*, macrophage-derived ROS induce Tregs, and this is suppressed by an NADPH-oxidase inhibitor (102). This finding was confirmed genetically, using macrophages from patients with CGD who have mutations in Nox2 or its key regulatory subunits resulting in either reduced expression or activity of the oxidase (102). Thus, CGD macrophages with mutated p47phox or Nox2 displayed reduced capacity for Treg induction and T cell suppression. Overall, it appears that Nox2 derived ROS may represent a potentially new target for therapeutic modulation of macrophage and Treg function (Figure 3), particularly in cancers that possess a strong inflammatory-based etiology.

ANTIOXIDANTS IN THE TREATMENT OF CANCER

There has been much interest in the use of antioxidants in the treatment of cancer given its potential to enhance treatment efficacy and/or reduce treatment side effects. While the diversity of agents and treatment paradigms investigated have resulted in a lack of clear evidence that such an approach will be beneficial, several lines of evidence suggest that this is an area that is worthy of further attention. With particular regards to angiogenesis and the signaling pathways already described in this review, it has been shown that antioxidants vitamins C and E reduce vascular VEGF and VEGFR-2 expression in apolipoprotein-E deficient mice (140). The thiol antioxidant *N*-acetylcysteine (NAC) inhibited endothelial invasion and sarcoma-induced angiogenesis *in vivo* (as well as inhibiting tumor take and

invasion and metastasis) (26). Similarly, pigment epithelium-derived factor, which has antioxidant properties, has been shown to inhibit angiogenesis and melanoma growth (2). HMG-CoA reductase inhibitors, statins, which have been shown to inhibit Rac1 activity (109) can block angiogenesis *in vivo* (196). The flavones and catechins are powerful flavonoids that can protect the body against reactive oxygen species. As dietary antioxidants, flavonoids have been associated with a lower incidence of carcinogenesis (41). Some flavonoids, such as fisetin, apigenin, and luteolin, are potent inhibitors of both tumor and endothelial cell proliferation (57), and suppress *in vitro* angiogenesis. While further research is required to determine the role for antioxidants in the management of cancer, it is conceivable that targeting NADPH oxidases and downstream signaling pathways may be a useful tool once suitable reagents are developed.

PHARMACOLOGICAL INHIBITORS OF NADPH OXIDASE

The evidence presented thus far provides a strong rationale for the future use of pharmacological inhibitors of NADPH oxidase to suppress the oxidative stress that triggers somatic mutations and macromolecular modifications during cancer, as well as the abnormal and persistent cell signaling pathways that underpin cell proliferation and apoptosis during angiogenesis. Currently, there are a number of inhibitors of NADPH oxidase displaying varying degrees of selectivity for the specific Nox isoforms. These drugs have been comprehensively reviewed recently (50, 165) and are provided in tabulated form in this review (Table 1). Despite the accumulating evidence that provides the rationale for pharmacological blockade of NADPH oxidase during cancer, currently there are very few studies that have tested such compounds using *in vivo* animal models. In order to inhibit Nox4 function pharmacologically *in vivo*, derivatives of the compound called fulvene, namely fulvene-5 have been used (19). To determine whether fulvene-5–induced loss of Nox4

demonstrated antitumorigenicity, mice were treated with fulvene-5 or vehicle control for 2 weeks, and tumor growth as well as tumor burden were analyzed thereafter. Tumor growth in mice treated with fulvene-5 was significantly reduced compared with that in vehicle control treated mice (19). Administration of plumbagin, which is generally considered an inhibitor of Nox4-dependent superoxide production, following ectopic implantation of hormone-refractory DU145 prostate cancer cells delayed tumor growth by 3 weeks and reduced both tumor weight and volume by 90% (9). Interestingly, cessation of plumbagin treatment for as long as 4 weeks did not result in progression of tumor growth. Although plumbagin has been touted as being a Nox4 specific inhibitor (53, 158), at concentrations as low as 5 micromol/L it was found to inhibit the expression of protein kinase C epsilon (PKC epsilon), phosphatidylinositol 3-kinase, phosphorylated AKT, phosphorylated janus-activated kinase-2, and phosphorylated signal transducer and activator of transcription 3 (Stat3) in both cultured prostate cancer cells and DU145 xenografts (9). These other actions are likely to have significant effects of tumor growth that are independent of Nox4.

CONCLUSIONS AND PERSPECTIVES

This review has summarized key current concepts and has touched upon some emerging ones regarding Nox signaling, as it pertains to angiogenesis in cancer. It has also highlighted the significant shortcomings in our current knowledge of this aspect of Nox signaling. As a consequence, substantial work remains to be performed to drive this exciting field forward. Indeed, future studies will need to carefully define: (1) stromal cell (i.e. endothelial cell)- and immune cell (macrophages, Tregs)- specific roles of Nox2 and other ROS generating enzymes including Nox1, Nox4, Duox1 and Duox2; (2) the activation mechanisms of NADPH oxidase by various (lymph)angiogenesis factors; and (3) molecular targets of NADPH oxidase-derived ROS in signaling pathways involved in

(lymph)angiogenic switch in different types of endothelial and cancer cells and (4) the X-ray crystal structures of catalytic (i.e. Nox) subunits and regulatory (i.e. p47phox, p67phox) subunits and their key binding sites to facilitate the development of more specific inhibitors of these enzyme complexes. The development of specific inhibitors of NADPH oxidases and redox signaling components (kinase, transcription factors and genes) could provide useful therapeutic strategies for treatment of various (lymph)angiogenesis-dependent pathologies during cancer.

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LIST OF ABBREVIATIONS

Angiopoietin-like-protein 4 (ANGPTL4)
 Chronic granulomatous disease (CGD)
 Extracellular regulated kinase (ERK)
 Flavin adenine dinucleotide (FAD)
 Hydrogen peroxide (H₂O₂)
 mitogen activated protein kinase (MAPK)
 Nitric oxide (NO)
 Peroxynitrite (OONO-)
 Phosphatidylinositol-3 kinase (PI3-K)
 polymerase δ -interacting protein 2 (POLDIP2)
 Reactive oxygen species (ROS)
 Vascular endothelial growth factor (VEGF)

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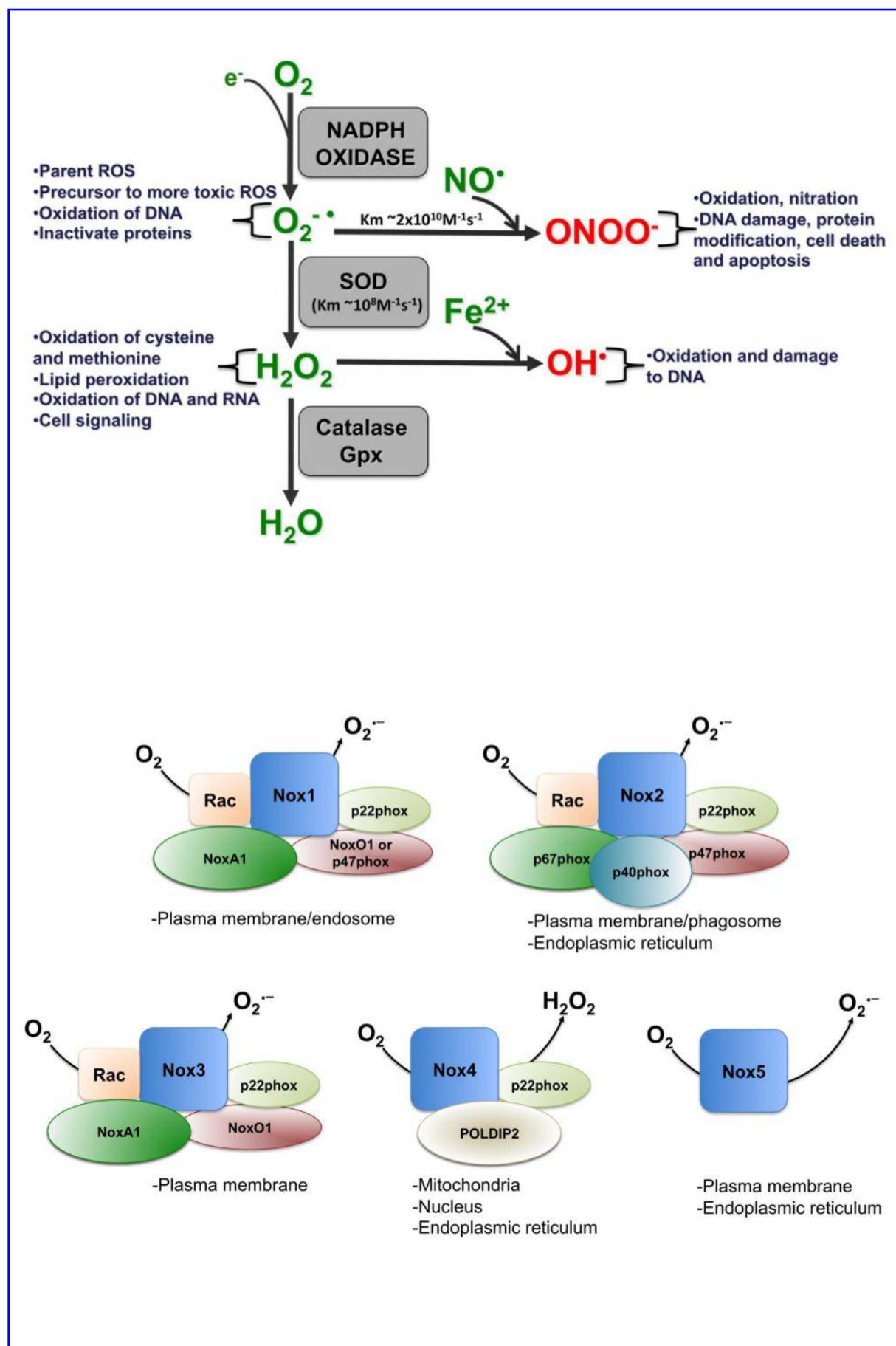
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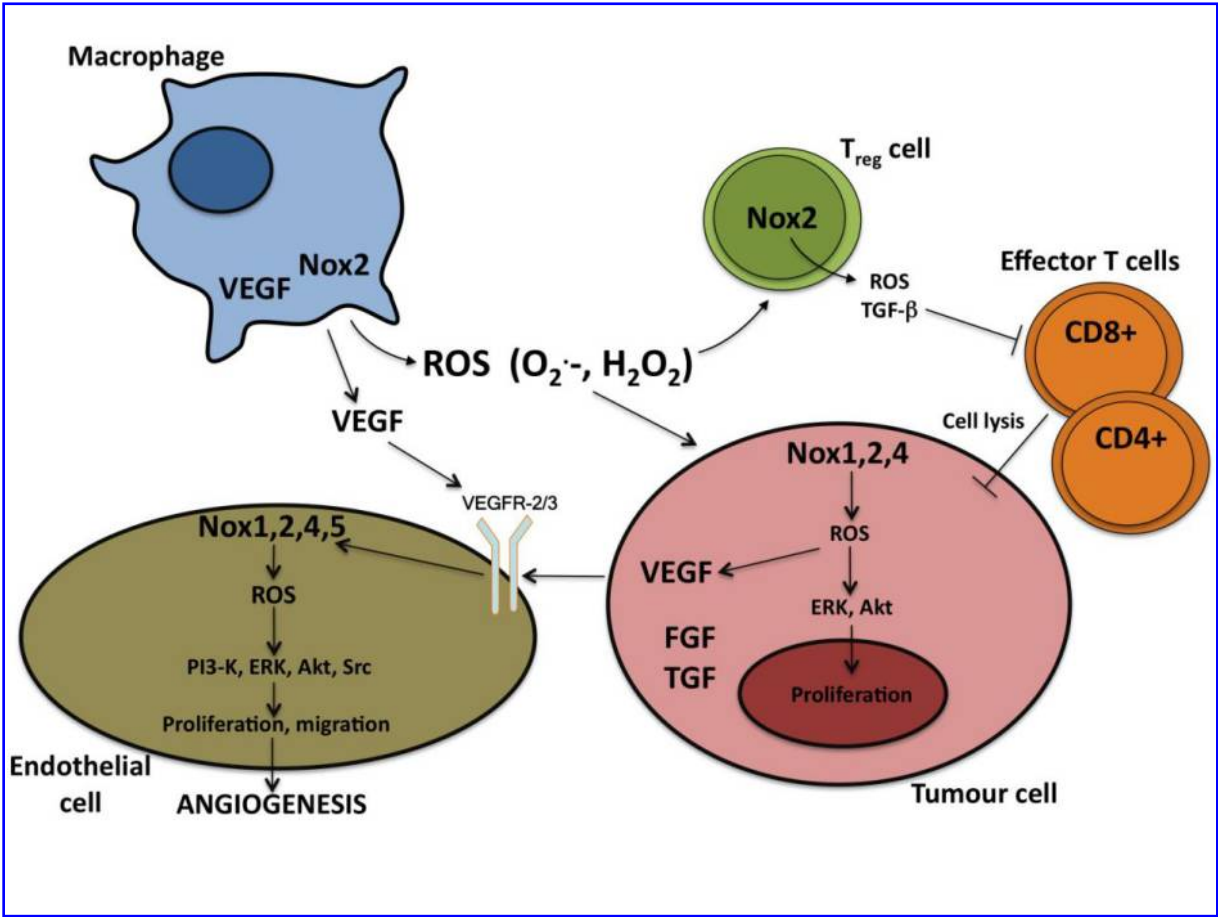
FIGURE LEGENDS

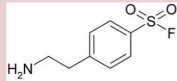
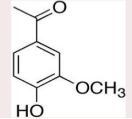
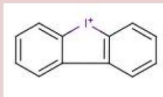

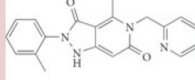
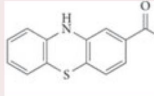
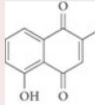
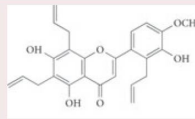
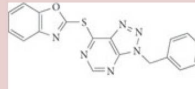
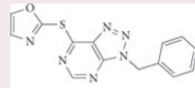
Figure 1. Pathways for the formation of ROS and selected biological effects.

Figure 2. Schematic diagram showing subunit composition of the 5 NADPH oxidase isoforms and their subcellular localization.

Figure 3. Schematic diagram depicting cellular localization of Nox within the tumor microenvironment as well as established and possible signaling pathways that underpin angiogenic responses. Growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and transforming growth factor- β (TGF- β) released by tumor cells and macrophages activate specific receptors on endothelial cells, which in turn leads to Nox recruitment and activation, ROS production, and proliferation and migration *via* extracellular signal-regulated kinase (ERK), PI3-K/protein kinase B (AKT) and Src pathways. Also, ROS produced within tumor cells influences their proliferation and the expression of VEGF-A. Moreover, macrophage Nox2-derived ROS may possess paracrine effects on T lymphocyte subsets such as CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) to promote their development and subsequent suppressive effects. The suppression of CD8⁺ and CD4⁺ effector T cells by Tregs may be partially due to Nox2-derived ROS. (VEGF, vascular endothelial growth factor; FGF, fibroblast growth factors; TGF- β , transforming growth factor- β ; ERK, extracellular-signal-regulated kinase; Akt, protein kinase b; PI3K, phosphoinositide-3-kinase).





Name	Chemical structure	Established or purported mechanism of action	Preferential Nox isoform(s) to be inhibited	Other pharmacological effects	Refs
AEBSF		Inhibits oxidase assembly by suppressing association of Nox2 with p47phox subunit	Nox2	Non selective protease inhibitor	(46)
APOCYNIN		Inhibits oxidase assembly by suppressing association of p47phox with membrane bound heterodimer	Nox2	H ₂ O ₂ scavenging	(171,176, 180)
DPI		Abstracts electrons from FAD and prevents electron flow through the flavocytochrome conduit	No selectivity demonstrated- inhibits all Nox isoforms	Inhibits NOS, NADH ubiquinone oxidoreductase, NADH dehydrogenase, xanthine oxidase, cytochrome P ₄₅₀ oxidoreductase	(35,65,177)
FULVENE		Fulvene-5 decreases Nox4- and Nox2 activity. Undefined mechanism.	Nox4 and Nox2	None reported	(19)
GK-136901		Undefined mechanism of action but similarity with NADPH suggests competitive substrate inhibitor	Nox1 and Nox4	None reported	(105,163)
ML171		Targets Nox1 subunit but not its cytosolic regulatory subunits (i.e. NoxO1, NoxA1 or Rac1).	Nox1	None reported	(70)
GP91 DSTAT	[H]-RKKRRQRRRCSTRIRRL-NH ₂	Inhibits oxidase assembly by inhibiting the association of Nox2 subunit with p47phox.	Nox2	None reported	(43,155)
PLUMBAGIN		Inhibits Nox4-dependent superoxide production in various cell lines that overexpress it. Mechanism of action unknown.	Nox4	Some ROS scavenger effects	(53,158)
PR-39	RRRPRPPYLPRRPPPFPP RLPPRIPPGFPPRFPPRFP	Binds to SH3 domains of the p47phox subunit and prevents binding to the p22phox subunit.	Nox2	Proteins with SH3 domains	(167)
S17834		Proposed to directly inhibit NADPH oxidase activity via undefined mechanism.	Nox2 and Nox4	None reported	(27)
VAS2870		Inhibits NADPH oxidase activity in Nox2 expressing cell lines. Undefined mechanism of action	Nox2 and Nox4	None reported	(182)
VAS3947		Reduces NADPH oxidase-derived ROS production in several cell lines irrespective of the specific isoforms.	Non selective	None reported	(199)